# Experiments on Green Algae Coexistent with Zooxanthellae in Sea Anemones[1]

LEONARD MUSCATINE[2]

ALONG THE PACIFIC COAST of North America sea anemones of the genus *Anthopleura* are dominant intertidal coelenterates. They occur as solitary tide pool inhabitants (*A. xanthogrammica*) or as aggregations carpeting firm substrates (*A. elegantissima*). One of the unique features of these species is their invariable symbiotic association with unicellular Dinophyceae. These brown or yellow-brown algae, known as zooxanthellae, occur intracellularly in the gastrodermal tissues of the host anemone (Muscatine, 1961).

The influence of these symbiotic algae on the biology of *Anthopleura* and on other anemones has been studied experimentally. The algae fix $^{14}CO_2$ and manufacture reduced organic carbon. Some of this carbon is translocated to the host anemone tissue where it is used in the synthesis of a wide range of animal constituents. In the absence of these algae, starved anemones lose weight faster than controls with algae. Thus, the adaptive value of the algae seems, in part, to be nutritional (Muscatine and Hand, 1958; Muscatine, 1961; Trench, 1968, 1969; Taylor, 1968, 1969). Within the past several years there have been unpublished reports concerning the occurrence of a green alga symbiotic with *Anthopleura* from the American Pacific coast. Since the natural coexistence of more than one species of symbiotic algae in a given host is rare, it seemed to be of interest to investigate this phenomenon. This study describes an attempt to define the symbiotic status of the green symbionts in *Anthopleura,* using criteria from known symbiotic associations involving zooxanthellae and sea anemones.

No attempt is made here to delve into the ecology of the associations. A precise description of the green symbiont awaits the work of a competent phycologist.

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[2] Department of Zoology, University of California, Los Angeles, California 90024.

## METHODS

All experiments were carried out on specimens of *Anthopleura elegantissima* (Brandt) collected at Cattle Point, San Juan Island, Washington, and on tentacles excised from *A. xanthogrammica* collected from the running seawater aquarium at the Friday Harbor Laboratories.

For histology, tissues were fixed in Bouin's fixative without acetic acid, embedded in paraffin (mp 58°–60° C), sectioned with a conventional microtome at 7 μ, and stained with Heidenhain's Mallory Azan (M. Natzler, personal communication).

For experiments with isolated algae, three to five tentacles of *Anthopleura xanthogrammica* were homogenized in a tissue grinder in 5 ml filtered seawater, or whole *A. elegantissima* were chilled to 5° C and homogenized in a Waring Blendor. Both procedures yielded a viscous green suspension which was centrifuged (International Clinical Centrifuge; 3,000 rpm) to yield a green pellet of algae and cell debris. The cells were washed twice in clean seawater by centrifugation and resuspension and a yield of about 0.25–0.5 ml wet-packed algae was obtained. The supernatant containing the homogenized animal tissue was saved and added back (1:1, v/v) to cell suspensions when incubation with host homogenate was required. In tracer studies, excised tentacles or suspensions of isolated algae (with or without homogenate added) were incubated in seawater with $Na_2$-$^{14}CO_3$ (10–50 μCi/ml) for 30 min to 3 hours at 12° C with illumination (1,500 ft-c) from two 100-watt incandescent bulbs. After incubation of the algae, the cells and medium were separated by centrifugation. Soluble labeled constituents of algae were extracted with hot 80 percent ethanol, assayed for radioactivity, and then analyzed by two-dimensional radiochromatography as previously described (Muscatine, 1965), following the original procedure of Benson et al. (1950). Alcohol-insoluble ma-

terial was resuspended in water and a portion was assayed for radioactivity.

For kinetic experiments on translocation, 6–12 tentacles of *A. xanthogrammica* were excised at their base with scissors and incubated with $Na_2{}^{14}CO_3$. After incubation, tissue layers were separated by lowering the pH of the seawater to pH 3.0 by adding HCl drop by drop. All of the tentacle ectoderm was then teased from the endoderm with Dumont no. 5 forceps coated with Beckman Desicote to prevent tissues from sticking to the dissecting tools. Each tissue was dried on a planchet and assayed for radioactivity.

### *Pigment Analysis*

Algae were extracted for pigment analysis according to the procedure of Haxo (personal communication). The extracts were chromatographed in one dimension on commercially prepared thin layer sheets of silicic acid (Chromar) in a dioxane-petroleum ether ascending solvent.

### *Identification of Photosynthetic Products of the Algae*

Soluble labeled compounds extracted from algae were eluted from chromatograms and cochromatographed with authentic compounds in three different solvents, as previously described (Muscatine, 1965).

### *Assay of Radioactivity*

Medium, alcoholic extracts, and alcohol-insoluble suspensions were assayed for radioactivity by placing 25-μl portions on nickel-plated planchets, adding a drop of glacial acetic acid to eliminate unused $Na_2{}^{14}CO_3$, and drying the planchet under an infrared lamp. Counts were made with a GM tube using a transistorized scaler (Nuclear Supplies, model SA-250). Counts were corrected for background and self-absorption. Separated tissues were dried on planchets, acidified, and counted.

## RESULTS

### *General Observations*

A survey of anemones from several collection sites on San Juan Island revealed that the green symbionts did not occur uniformly amongst all anemone populations (Table 1). *Anthopleura elegantissima* collected at False Bay contained only the conventional zooxanthellae. Those from Cattle Point harbored mixed populations of zooxanthellae and zoochlorellae with the green algae predominating. Excised tentacles of some specimens of *A. elegantissima* appeared to contain only green algae, but a thorough inspection of all tissues was not undertaken. A single field specimen of *A. xanthogrammica* was sampled and found to contain zooxanthellae predominantly, with occasional green algae (Fig. 1), while another specimen of this species which had been kept in the Friday Harbor Marine Laboratory aquarium system for more than a year contained green symbionts exclusively (Fig. 2). This may represent a special case, where prolonged existence under aquarium conditions may have affected the make-up of the symbiont population.

TABLE 1

SUMMARY OF COLLECTION SITES AND SYMBIONT POPULATIONS IN *Anthopleura* SPP. FROM SAN JUAN ISLAND, WASHINGTON

| SPECIES OF ANEMONE | COLLECTION SITE | SYMBIONT TYPES |
|---|---|---|
| *A. elegantissima* | False Bay | zooxanthellae only |
| *A. elegantissima* | Cattle Point | zooxanthellae and zoochlorellae together, with latter most abundant |
| *A. elegantissima* | Cattle Point | zoochlorellae only |
| *A. xanthogrammica* | Eagle Cove (single specimen) | zooxanthellae and zoochlorellae, with the former most abundant (See Fig. 2) |
| *A. xanthogrammica* | Friday Harbor Laboratories Aquarium | dense populations of zoochlorellae (See Fig. 2) |

*A. elegantissima* with mixed populations of symbionts were the most common type of anemone at Cattle Point. To determine the approximate proportion of each type of algae residing in these individuals, tentacles were sampled from several anemones and with a glass rod the algae were expressed onto a glass slide. About 10 separate slide fields were photographed in color and, from these, counts were made of each

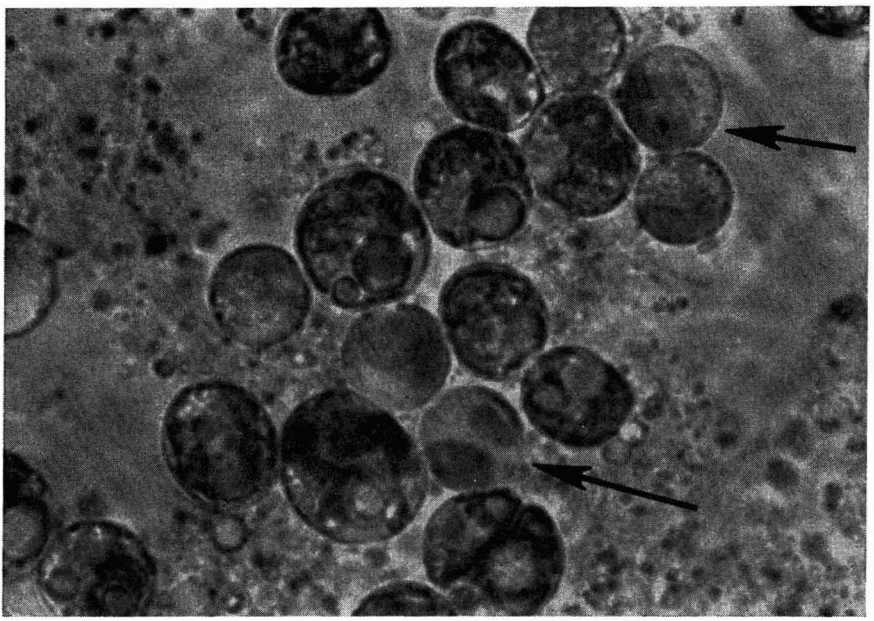

FIG. 1. Mixed algae, predominantly brown, from a tentacle of *Anthopleura xanthogrammica* collected from Eagle Cove. Arrows indicate zoochlorellae. (960 ×)

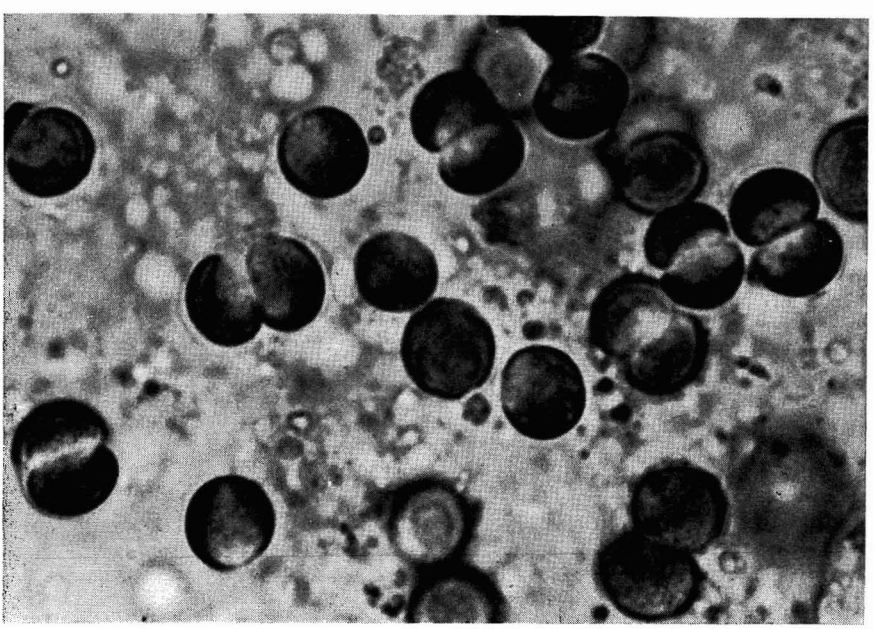

FIG. 2. Exclusively green algae from *Anthopleura xanthogrammica* maintained in Friday Harbor Laboratories' aquarium. (960 ×)

algal type. In a total sample of 700 cells, the ratio of green to brown algae was 621:79, or about 8:1.

Observations were also made on the number of cells exhibiting a distinct equatorial furrow, this being a criterion for whether or not a cell was dividing. Zooxanthellae were expressed from tentacles of *A. elegantissima* which contained this algal type exclusively. Zoochlorellae were obtained from tentacles of *A. xanthogrammica.* Algae in six separate microscope fields were counted. Of the zoochlorellae (36/161) counted, 18 percent were dividing, while 5.5 percent of the zooxanthellae (16/272) were dividing. These observations are not interpreted as conclusive but merely indicative, since they represent a small sample and they do not account for fluctuations in rate of division as a function of host nutrition, season, etc.

Zooxanthellae normally occur exclusively in the gastrodermis of *Anthopleura* and in other marine anthozoans. Examination of serial sections of tentacles of *A. xanthogrammica* containing only green symbionts, and of *A. elegantissima* containing mostly green symbionts, revealed that the algae were situated only in the gastrodermis. Although they appeared to be intracellular, electron microscopy and maceration would be required to verify this.

The green symbionts are single spherical cells, 6–10 μ in diameter, with a smooth surface and a rich green coloration. The chloroplast is simple, peripheral, and cup-shaped. Cells in division exhibit a single meridional furrow.

Pigment analyses (Fig. 3) of green and brown symbionts showed clearly the expected differences in chlorophyll and carotenoid contents of the two cell types. The pigments of zooxanthellae are now well characterized (Taylor, 1967; Jeffrey and Haxo, 1968) by the presence of chlorophylls *a* and *c* and the xanthophyll peridinin. Chromatographic analysis of the green algal pigments revealed chlorophylls *a* and *b* and several unidentified xanthophylls. This pigment suite is consistent with the interpretation that the algae are Chlorophyceae but by itself does not constitute absolute proof. Similar pigments were observed by Hartman (unpublished) in analyses of green algae from *A. xanthogrammica.*

*Experimental*

A wide range of symbiotic algae displays characteristics which do not occur in free-living algae from the same or related taxa (Smith, Muscatine, and Lewis, 1969). These experimentally defined characteristics are: (1) Symbiotic algae, when isolated from the host and incubated in vitro, manufacture soluble photosynthetic products and selectively release to the environment a significant fraction of these, usually as carbohydrate; (2) Release of soluble products of photosynthesis by symbiotic algae is susceptible to control by various factors. For example, zoochlorellae from freshwater hosts excrete maltose only when environmental pH is in the acid range (Muscatine, 1965; Smith, Muscatine, and Lewis, 1969). Similarly, the level of excretion of glycerol by zooxanthellae in vitro is significantly increased by the presence, in the incubation medium, of a homogenate of host tissue (Muscatine, 1967; Trench, 1969; Taylor, 1969); (3) Release of photosynthetic products and translocation to host tissues can be detected in vivo. All of these characteristics have been observed in zooxanthellae from *A. elegantissima.*

Trench (1969) showed that isolated zooxanthellae from *A. elegantissima* fix $^{14}CO_2$ and selectively release glycerol-$^{14}C$ to the external medium; that excretion is enhanced by incubating algae with host homogenate; and that translocation can be demonstrated in vivo.

To determine the nature of the photosynthetic products of the green symbionts, and whether or not the symbionts release soluble products in vitro, algae were isolated and incubated with $Na_2{}^{14}CO_3$ as described. In addition, some were incubated with host homogenate to determine if excretion was dependent on, or enhanced by, host homogenate. After incubation, cells and medium were separated, cells were extracted with ethanol, and the soluble fractions assayed for radioactivity and analyzed chromatographically. Table 2 shows the results of in vitro excretion by green symbionts compared with the data of Trench (1969) for zooxanthellae. Green algae isolated from *A. xanthogrammica* and *A. elegantissima* fix an appreciable amount of $^{14}CO_2$. Chromatographic analysis shows that

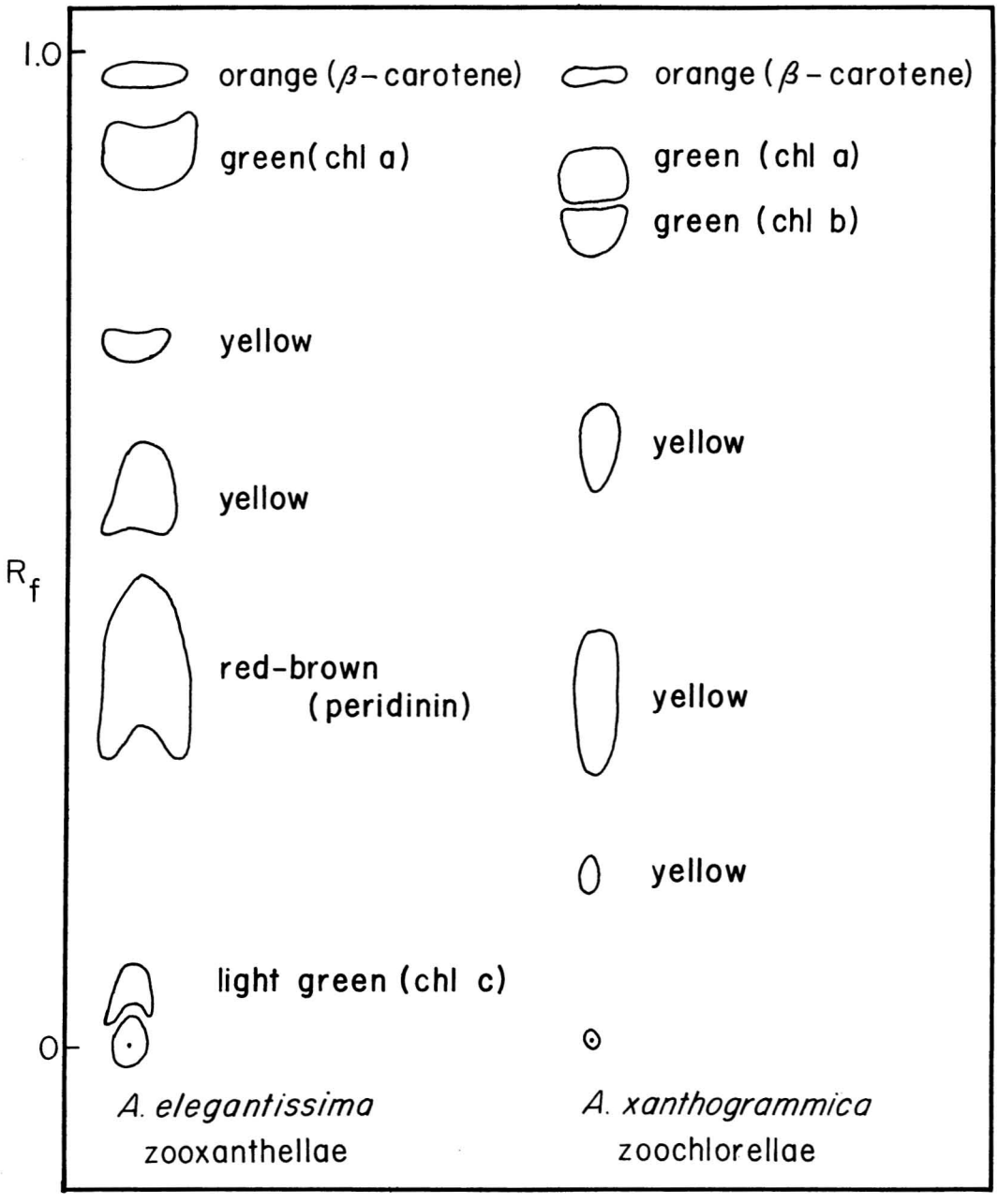


FIG. 3. Chromatogram of pigments of zooxanthellae (left) and zoochlorellae (right) from *Anthopleura*. Pigments extracted in absolute methanol, transferred to diethyl ether, and applied to commercially prepared silicic acid thin layer sheets (Chromar 500). Developed in 15 percent hexane in petroleum ether. Identifications in parentheses are tentative, based on comparison with $R_F$ values of known pigments.

TABLE 2

PHOTOSYNTHESIS AND RELEASE OF $^{14}C$ BY ALGAE FROM *Anthopleura* SPP. INCUBATED IN VITRO

| SPECIES FROM WHICH ALGAE OBTAINED | WITH ANIMAL HOMOGENATE | | | WITHOUT ANIMAL HOMOGENATE | | |
|---|---|---|---|---|---|---|
| | CELLS (cpm) | MEDIUM (cpm) | $^{14}C$ RELEASED (%) | CELLS (cpm) | MEDIUM (cpm) | $^{14}C$ RELEASED (%) |
| *A. xanthogrammica* (green) | 515,580 | 6,000 | 1.1 | 309,500 | 1,350 | 1.0 |
| *A. elegantissima* (green) | 270,270 | 3,100 | 1.1 | 151,140 | 3,180 | 2.0 |
| *A. elegantissima* (green and brown) | 626,200 | 31,300 | 4.7 | 457,650 | 21,280 | 4.4 |
| *A. elegantissima** (brown) | 65,766 | 75,400 | 58.0 | 9,072 | 5,320 | 31.0 |

NOTE: About 0.5 ml wet-packed cells incubated in 1.5 ml seawater plus 1.5 ml host homogenate; 1,500 ft-c; 20 $\mu$Ci $Na_2{}^{14}CO_3$; 1 hour; 12° C.

* Data from Trench (1969, p. 101). About 0.1 ml wet-packed algae in 2 ml homogenate; 500 ft-c; 5 $\mu$Ci/ml $NaH^{14}CO_3$; 1 hour; 18° C.

sucrose is the major labeled soluble intracellular product. However, only a fraction of the total $^{14}C$ fixed by the cells (less than 2.0 percent) can be detected in the medium after an hour of incubation regardless of whether the algae were incubated with or without host homogenate. This level of excretion, too low to be conclusively analyzed chromatographically, is characteristic of free living algae which normally release small amounts of organic material to the medium (Hellebust, 1965). A slightly higher level of excretion was observed in the medium of the suspensions of mixed symbionts. Excretion by zooxanthellae, which constitute approximately 10 percent of the population, may account for this. The data of Trench (1969) for *A. elegantissima* contrast sharply with those of symbiotic zoochlorellae. Zooxanthellae in seawater without added host homogenate also fix $^{14}C$ but excrete about 30 percent of the total $^{14}C$ to the medium. If host homogenate is added, excretion levels nearly double to 58.0 percent. In this instance chromatographic analysis shows several discrete labeled products in the medium, with glycerol as the major product. The major intracellular product was identified as glucose.

These observations suggest that the isolated green symbionts do not release significant amounts of labeled material to the medium. In this respect, they behave more like free-living algae than conventional symbionts. However, since conditions in vitro might not accurately reflect conditions in vivo, it seemed desirable to investigate the possibility that the green algae might translocate photosynthetic products to the host in vivo, even though, in most translocation systems involving autotrophic symbionts, in vivo movement of carbon is demonstrable in vitro as well (Smith, Muscatine, and Lewis, 1969). To ascertain this, excised tentacles of *A. xanthogrammica,* which contained only green symbionts, were incubated with $Na_2{}^{14}CO_3$ for up to 3 hours. Periodically, a sample tentacle was rinsed and then treated to quantitatively separate ectoderm and endoderm. Mesoglea was retained with the endoderm. Each tissue was then assayed for radioactivity. Data given in Table 3 for the zoochlorellae are compared with data from a similar experiment by Trench (1969) on tentacles with zooxanthellae from *A. elegantissima.* The data for green algae show most fixation of $^{14}C$ in the gastrodermis where the algae are located. $^{14}C$ detected in ectoderm was only a small percentage of the total $^{14}C$ fixed, ranging from 2.7 to 3.6 percent. In contrast, of the total $^{14}C$ fixed by zooxanthellae in tentacles of *A. elegantissima,* a significant fraction (25–38 percent) was detected in the algae-free ectoderm. Trench analyzed this ectoderm and showed that $^{14}C$ was incorporated into several host tissue fractions. Low levels of $^{14}C$ in controls incubated in darkness ruled out the possibility of significant heterotrophic fixation of $^{14}C$ by the ectoderm. These data are interpreted as demonstrat-

TABLE 3

DISTRIBUTION OF $^{14}C$ IN TENTACLES FROM *Anthopleura* SPP. INCUBATED IN LIGHT WITH $^{14}CO_2$

| SPECIES FROM WHICH ALGAE OBTAINED | TIME (min) | GASTRODERM (cpm) | ECTODERM (cpm) | $^{14}C$ IN ECTODERM (%) |
|---|---|---|---|---|
| *A. xanthogrammica* (green) | 30 | 9,290 | 260 | 2.7 |
| | 60 | 9,790 | 316 | 3.1 |
| | 110 | 20,700 | 600 | 2.8 |
| | 140 | 19,850 | 750 | 3.6 |
| | 170 | 18,150 | 525 | 2.8 |
| | | (dpm/mg N) | (dpm/mg N) | |
| *A. elegantissima** | 60 | 5,100 | 3,131 | 38.0 |
| | 90 | 12,855 | 4,285 | 25.0 |
| | 180 | 16,476 | 6,093 | 27.0 |

NOTE: Five tentacles in 3 ml seawater; 50 $\mu$Ci $Na_2{}^{14}CO_3$; 1,500 ft-c; 12° C.
* Data from Trench (1969, p. 17). 5 $\mu$Ci/ml; 900 ft-c; 18° C.

ing that zooxanthellae fix carbon and translocate $^{14}C$ in vivo to the ectoderm. In comparison, the zoochlorellae appear to fix $^{14}C$ but $^{14}C$ translocated to the ectoderm was only a small fraction of the total $^{14}C$ fixed. It is concluded that the green algae do not translocate significant carbon to the ectoderm in vivo, at least under the conditions of the experiment.

## DISCUSSION

The results of this investigation show that the subsidiary algae in *Anthopleura* are green algae whose metabolism of $^{14}CO_2$ differs in several respects from that of zooxanthellae. The green symbionts fix an appreciable amount of $^{14}CO_2$, but neither release a significant amount of soluble $^{14}C$-labeled organic material to the medium when incubated in vitro with or without host homogenate, nor do they appear to translocate appreciable amounts of labeled compounds from algae to epidermis in isolated tentacles. The data do not rule out the possibility that in vivo translocation might be restricted to the gastrodermis. Since appreciable levels of release of $^{14}C$ (as glycerol-$^{14}C$), especially in the presence of host homogenate, can be demonstrated in experiments with isolated zooxanthellae and since significant translocation to ectoderm can be detected in vivo, it is concluded that, if the green algae function as mutualistic symbionts, their biochemical interactions are distinct from those seen in the association of anemones and zooxanthellae. The question is raised whether the green algae interact with the metabolism of *Anthopleura* in other ways which may be interpreted as "mutualistic" which cannot be detected by the methods used here. For example, there is the ever-present, but rarely tested, hypothesis that the host is digesting and "farming" the algae, a process which might occur only in the gastrodermal digestive zones of the mesenteries and not in the tentacles.

The absence of significant excretion of $^{14}C$-labeled metabolites by the green algae might also be reconciled if it is assumed, from the data on frequency of cell division, that the green algae are growing relatively rapidly and therefore do not mobilize excess soluble material for release to the host. Rapid growth of the algae would pose no problem if the anemone could rid itself of excess algae, as has been claimed by Taylor (1969) for *Anemonia sulcata.*

The occurrence of subsidiary algae in *Anthopleura* might be expected on the grounds that this anemone is competent to acquire and maintain algae within its cells, the persistence of potential symbionts being governed by an as-yet-unknown set of interactions between algae and host. Artificial infections of *Paramecium bursaria* and *Hydra* with free-living algae showed that the free-living algae gain access to the host cells but do not persist to the same extent as natural symbionts (Karakashian and Karakashian, 1965; Park, Greenblatt, Mattern, and Merril, 1967). Free-living *Platymonas* will infect and persist in *Convoluta rescoffensis* but, when the natural symbiont is introduced, it

gradually replaces the artificially introduced symbiont (Provasoli, Yamasu, and Manton, 1968). Thus the green algae in *Anthopleura* may have properties which are required by algae to gain access to, and persist in, the host. This suggests that the algae may differ from their free-living counterpart in that the symbionts are not digested by, or rejected from, the host. The extent of any competition between green and brown symbionts has not yet been ascertained. On the other hand, the green algae closely resemble a species which has been observed in the epidermis of certain starfish (*Henricia, Solaster*) from Puget Sound (R. Norris, University of Washington, personal communication) and is believed to cause the death of these animals. With this in mind, the occurrence of such green algae in *Anthopleura* may represent an attempt by the algae to establish a symbiotic (parasitic?) relationship with the anemone. The alga can gain access to the anemone because the animal takes particulate food into its digestive gastrodermis by phagocytosis. The anemone, however, unlike the starfish, may inhibit very rapid growth of the symbiont, or may easily rid itself of excess algae, thus offsetting the possibility of a lethal parasitic infection. Ultimately, definition of the role of the algae will require experimental observations on the well-being of the host.

SUMMARY

1. Green algae coexist with zooxanthellae as symbionts in sea anemones of the genus *Anthopleura.* Observations were made on their numbers and local occurrence on San Juan Island, Washington.

2. These green algae neither release soluble $^{14}C$-labeled photosynthetic products to the medium in vitro nor translocate labeled compounds to the tissues of the host. In this respect they differ from zooxanthellae, which selectively release glycerol in vitro and translocate labeled compounds to the host tissue.

3. The possible *raison d'être* of this mixed symbiosis is discussed. It is speculated that, although the green algae can enter the host, a potentially lethal "parasitic" infection is offset by the ability of the host to regulate numbers of symbionts, either natural or introduced, to a compatible level.

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